

Increased levels of E2F-1-dependent DNA binding activity after UV- or γ -irradiation

Manuela Höfferer, Christiane Wirbelauer, Bostian Humar¹ and Wilhelm Krek*

Friedrich Miescher Institut, Maulbeerstrasse 66, CH-4058 Basel, Switzerland and ¹Departement Forschung, Kantonsspital Basel, Hebelstrasse 20, CH-4031 Basel, Switzerland

Received October 2, 1998; Revised and Accepted November 13, 1998

ABSTRACT

In mammalian cells, DNA damage induces robust changes in gene expression and these changes contribute to the proper execution of cellular responses to DNA damage, including DNA repair, cell cycle arrest and apoptosis. The transcription factor E2F-1 has been suggested to play a key role in the regulation of cell cycle-dependent gene expression and apoptosis. These activities depend on the ability of E2F-1 to form functionally active DNA binding complexes. Here we describe an assay that allows one to measure E2F-1 DNA binding activity in naive cells. We find that DNA damage, generated by UV- or γ -irradiation, prompts increased production of E2F-1 DNA binding activity, which, at least in part, originates from alterations in E2F-1 protein levels. These findings represent an indication for a role of the transcription factor E2F-1 in the DNA damage response pathway.

INTRODUCTION

Mammalian cells have evolved a complex, multifaceted network through which DNA damage is detected and an appropriate response is implemented (1). These cellular responses to damaged DNA represent defenses against genomic instability and tumorigenesis resulting from unrepaired damage (2). DNA damage inflicted by radiation (or by genotoxic drugs) is detected by a dedicated DNA damage-sensing apparatus which then generates a signal that can lead to DNA repair, cell cycle arrest and apoptosis. Which fate prevails depends on the severity of the damage and the type of cell affected. The signal transduction system, which communicates information between a DNA lesion and components of the cell cycle, is referred to as the DNA damage checkpoint (1).

In mammalian cells, the p53 tumor suppressor is the archetype of known DNA damage checkpoint regulators. p53 is stabilized and activated as a transcription factor following genomic DNA damage and under such circumstances provides a crucial block to cell cycle progression, leading to growth arrest (to allow time for repair processes) or apoptosis (3). Because p53 is mutated in a large fraction of cancers of diverse types, it is thought that the tumorigenic process may be intimately related to the disruption of p53-mediated control of the cell cycle. The precise signal transduction pathway that senses DNA damage and recruits p53 has not been elucidated, but it is likely to include ATM, a member

of the PI3 kinase-related protein kinase superfamily, as well as other proteins known to be critical regulators of the cellular response to DNA damage, such as c-abl and CHK1 (1).

E2F, a family of heterodimeric transcription factors composed of E2F-like and DP-like subunits, is crucial for transcriptional activation of genes that regulate S phase entry and genes that function to engage DNA synthesis (4). E2F-1, the first cloned member of this family, plays a key role in the regulation of cell proliferation and apoptosis. Overexpression of E2F-1 can cause oncogenesis (5–7) and is sufficient to drive serum-starved fibroblasts into S phase (8–11). In some settings, prolonged expression of E2F-1 drives cells also to undergo p53-dependent or p53-independent apoptosis (9–15). Recent observations, derived from studies on mice homozygous for a non-functional E2F-1 allele, strongly suggest that E2F-1 has a tumor suppression function as well, which might be linked to its apoptosis-inducing activity (16,17). Despite the evidence that E2F-1 is a key participant in the regulation of cell cycle progression and apoptosis, no information exists about a potential sensitivity of E2F-1 activity to DNA damage. Here we report on an assay that we have developed allowing one to monitor the DNA binding activity of endogenous E2F-1. We find that two distinct DNA damaging agents, UV- and γ -irradiation, trigger the production of increased amounts of DNA binding competent E2F-1, implying that E2F-1 participates in the DNA damage response pathway.

MATERIALS AND METHODS

Cell culture, radiation treatment and cell cycle analysis

Human U2-OS and T98G cells were maintained and synchronized by serum deprivation as described previously (18). At indicated time points after release from serum deprivation, tissue culture dishes receiving radiation treatment were exposed to UV-C (Stratalinker™ 1800 UV crosslinker; Stratagene, La Jolla, CA) or γ -rays (using a ⁶⁰Co source). For UV-irradiation of cells, the culture medium was aspirated, cells were washed once with phosphate-buffered saline (PBS) and uncovered tissue culture plates were irradiated with 20 J/m². Afterwards, culture medium was added back to the cells. For γ -irradiation, cells were directly exposed to a dose of 6 Gy, without removing the medium. For control, the same procedure was followed as described above, except that cells were not exposed to radiation. Typically, cells were 50–80% confluent at the time of irradiation and were harvested 1 h after treatment by trypsinization. Cell cycle progression was monitored by flow cytometry as described (18).

*To whom correspondence should be addressed. Tel: +41 61 697 8620; Fax: +41 61 697 3976; Email: wilhelm.krek@fmi.ch

Antibodies

For the production of peptide antibodies recognizing human E2F-1, the peptide ALGRPPVKRRDLTDHQYL, corresponding to a region in E2F-1 spanning amino acid residues 82–101, was synthesized. The peptide was coupled to keyhole limpet hemocyanin (Pierce) by glutaraldehyde coupling and injected into rabbits. Anti-peptide antibodies were affinity purified by coupling 10 mg peptide to 1 g CH-Sepharose 4B (Pharmacia) according to the manufacturer's protocol. Incubation and elution of the antibody, referred to as anti-E2F-1(N), was carried out as described (18). The mouse monoclonal antibody (mAb) E2F-1 Powerclonal™ (PC), recognizing human E2F-1, was purchased from Upstate Biotechnology. The affinity purified rabbit polyclonal peptide antibodies E2F-1(C20)X [referred to in the manuscript as anti-E2F-1(C)] and E2F-4(C20) recognizing the C-termini of human E2F-1 and E2F-4, respectively, were obtained from Santa Cruz Biotechnology. Anti-cyclin A antibodies raised against full-length human cyclin A have been described previously (18).

Whole cell extracts and immunoblotting

For whole cell extract preparation, cells were washed twice in PBS and lysed in extraction buffer (20 mM HEPES, pH 7.9, 0.5% NP-40, 1 mM EDTA, 0.4 M NaCl, 25% glycerol, 10 mM NaF, 0.1% NP-40, 1 mM DTT, 1 mg/ml aprotinin, 1 mM PMSF) for 30 min on ice. Extracts were clarified by centrifugation and quantitated with the BioRad protein assay reagent kit. Immunoblotting was performed according to published procedures (18). Antibody detection was achieved by enhanced chemiluminescence (ECL; Amersham).

Immunoprecipitation, peptide release assay and EMSA

For immunoprecipitation, 80% confluent T98G or U2-OS cells in a 10 cm dish were lysed in 800 µl TNN buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.5% NP-40, 1 mM DTT, 1 mg/ml aprotinin, 1 mM PMSF) and incubated on ice for 30 min. Extracts were clarified by centrifugation, quantitated with the BioRad protein assay reagent kit and equalized amounts of cell protein were subjected to immunoprecipitation with 1.5 µg of the indicated antibodies. For peptide competition experiments, antibodies were preincubated with 2 µg of the corresponding peptide for 15 min at 4°C. Protein A-Sepharose was added, immune complexes were collected by centrifugation and washed four times with TNN buffer and once with gel shift buffer (20 mM HEPES, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 10% glycerol, 0.1% NP-40, 0.1 mM EDTA, 0.5 mM DTT). To disrupt antibody-antigen interactions, washed immune complexes were incubated with gel shift buffer containing 2 mg/ml competing peptide at 4°C for 10 min. Subsequently, samples were centrifuged at 14 000 g at 4°C for 10 min, supernatants were collected, transferred into fresh Eppendorf tubes and directly used in electrophoretic mobility shift assays (EMSAs) (19). For antibody supershifts, 1 µg of indicated antibodies were added to binding reactions prior to the addition of labeled E2F oligonucleotide. For competition, a 100-fold molar excess of unlabeled E2F oligonucleotide was added to the gel shift assay. The sequence of the E2F oligonucleotide corresponds to the DHFR promoter and has been described (19).

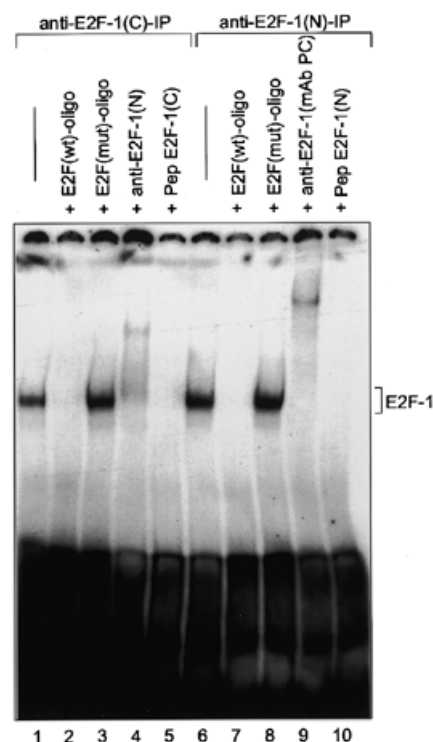


Figure 1. Measurement of endogenous E2F-1 DNA binding activity. T98G cells were growth arrested by serum deprivation and stimulated to re-enter the cell cycle by serum addition. Twenty two hours after serum stimulation (corresponding to early S phase), whole cell extracts were prepared and processed for immunoprecipitation with either anti-E2F-1(C) antibody (lanes 1–5) or anti-E2F-1(N) antibody (lanes 6–10) in the absence (lanes 1–4 and 6–9) or presence (lanes 5 and 10) of the corresponding immunizing peptides. Immune complexes were collected with protein A-Sepharose and treated with the corresponding immunizing peptides, as indicated at the top of the figure, to interrupt antibody-antigen interaction. The resultant supernatants were analyzed by EMSA assay for E2F DNA binding activity in the presence of either anti-E2F-1(N) antibody (lane 4) or anti-E2F-1 mAb PC (lane 9). Competition experiments were done with oligonucleotides containing either a wild-type (lanes 2 and 7) or a mutant (lanes 3 and 8) E2F site derived from the dihydrofolate reductase (DHFR) promoter.

RESULTS AND DISCUSSION

The abundance of E2F-1 is relatively low in multiple cell lines and hence, it has proven difficult in the past to measure specifically E2F-1 DNA binding activity in whole cell extracts without the use of transfection technology. We attempted to devise an assay that allows one to monitor selectively E2F-1 DNA binding activity. To this end, anti-E2F-1 immunoprecipitates were generated from whole cell extracts derived from T98G cells that had been previously synchronized in late G₁/early S phase (a time where E2F-1 expression is highest) using peptide antibodies raised to distinct epitopes of human E2F-1. Antibody-antigen interactions were resolved by treatment of immune complexes with the relevant immunizing peptide (referred to as C-peptide and N-peptide) and supernatants were tested in gel shift assays. As shown in Figure 1, anti-E2F-1(C) or anti-E2F-1(N) immunoprecipitates contained E2F gel shift activity (lanes 1 and 6, respectively). This activity was absent when the C- or N-peptide was added to the corresponding antibody prior to immunoprecipitation

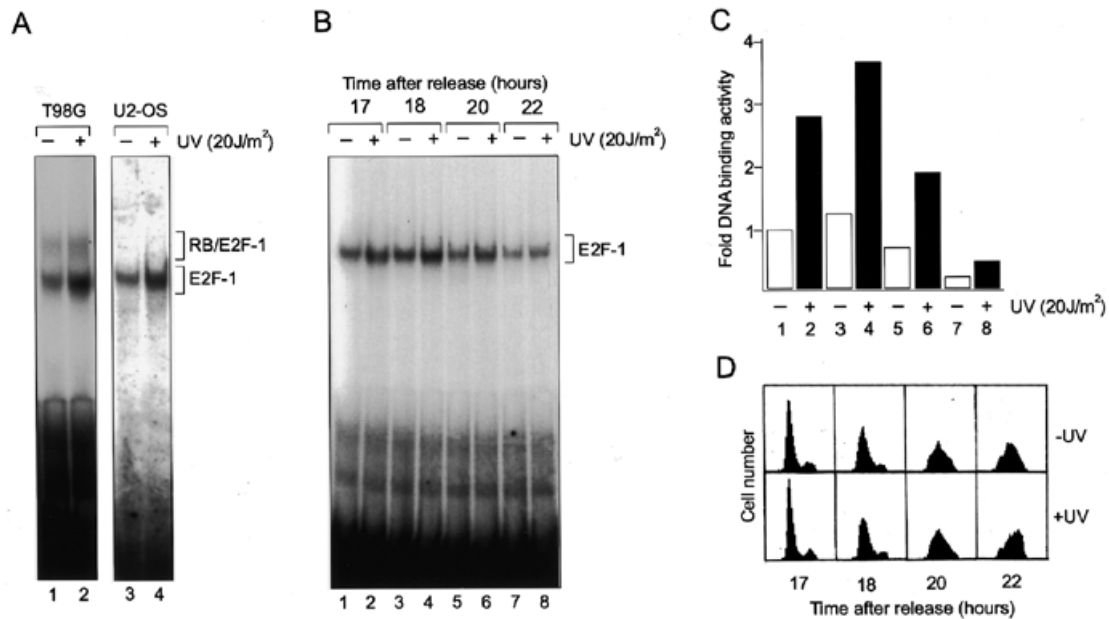


Figure 2. Effect of UV-irradiation on E2F-1 DNA binding activity. (A) Exponentially growing T98G or U2-OS cells were either left untreated (lanes 1 and 3) or were treated with 20 J/m² UV-irradiation (lanes 2 and 4) followed by an incubation period of 1 h. Whole cell extracts were prepared and processed for E2F-1 IP gel shift assay as described in Figure 1 using anti-E2F-1(C) antibody. Note the increase in immunoprecipitable E2F-1-specific DNA binding activity in response to DNA damage. (B) T98G cells were growth arrested by serum deprivation and stimulated to re-enter the cell cycle by serum addition. At indicated times after serum stimulation, cells were either left untreated (lanes 1, 3, 5 and 7) or were treated with the indicated dose of UV (lanes 2, 4, 6 and 8). Whole cell extracts were prepared, corrected for total protein amounts and processed for E2F-1 IP gel shift assay immunoprecipitation with anti-E2F-1(N) antibody. The autoradiogram shown is a representative of multiple independently performed experiments. (C) E2F-1 gel shift activities shown in (A) were densitometrically traced. The E2F-1 DNA binding activity detected at 17 h after serum addition (lane 1) was set to 1 and used to normalize the signal at the subsequent time points. (D) Cells harvested in (A) either before (upper panel) or after (lower panel) UV-irradiation were fixed in 70% ethanol, stained with propidium iodide and subjected to flow cytometric analysis to determine cell cycle distribution.

(lanes 5 and 10, respectively). Competition experiments indicated that the observed DNA binding activity was E2F-specific (Fig. 1, lanes 2, 3, 7 and 8). Moreover, the recovered E2F gel shift activity was supershifted by anti-E2F-1(N) antibody (lane 4) or by anti-E2F-1 mAb PC (lane 9). Taken together, these data suggest that the procedure employed here permits the specific and selective recovery of E2F-1 DNA binding activity from whole cell extracts of naive cells. For simplicity we refer to this whole procedure as the E2F-1 immunoprecipitation (IP) gel shift assay. The absence of a RB/E2F-1 gel shift complex can be explained by the fact that in this particular experiment early S phase synchronized T98G cells were used. At that time pRB is hyperphosphorylated and unable to bind E2F-1. Using the very same procedure, we did, however, recover an RB/E2F-1 gel shift complex from asynchronously growing T98G cells (Fig. 2A, lanes 1 and 2).

There is abundant evidence that E2F-1 plays a critical role in normal cell cycle regulation. However, there is little evidence that indicates a role of E2F-1 in the cellular response to DNA damage. Thus, we asked whether the DNA binding activity of E2F-1 changes in response to DNA damage. Exponentially growing T98G and U2-OS cells were exposed to UV at a dose of 20 J/m² and harvested 1 h later. E2F-1 IP gel shift analysis of unperturbed control cells revealed the presence of one major, faster migrating band corresponding to 'free' E2F-1 (Fig. 2A, lanes 1 and 3) and one minor, slower migrating band (Fig. 2A, lane 1). The slower migrating gel shift activity contains pRB, since it was specifically supershifted by anti-pRB antibody (data not shown). The absence of an RB/E2F-1 gel shift activity in U2-OS cells is likely due to

the fact that exponentially growing U2-OS cells contain little, if any, underphosphorylated pRB. Strikingly, UV treatment led to a significant increase in E2F-1 immunoprecipitable gel shift activity in both cell lines (Fig. 2A, lanes 2 and 4). The simplest model to explain this observation would be one in which DNA damage, produced by UV, triggers a signaling cascade, one outcome of which is increased levels of E2F-1 DNA binding activity.

The expression of E2F-1 protein is periodic, peaking in early/mid S phase and then decaying as cells progress into the late S/G₂ phases (20). Thus, we asked whether UV can effect E2F-1 DNA binding activity at all times during G₁ and S phase progression. To this end, T98G cells were synchronized by serum deprivation in G₀/G₁, released from the arrest by the addition of serum and at indicated time points IP gel shift assays were performed, in parallel, from untreated cells or from cells treated with UV at a dose of 20 J/m² as a stimulus. Cell synchrony was monitored in parallel by flow cytometry (Fig. 2D). Cells released into early S phase (17 and 18 h after serum addition) contained significant amounts of E2F-1 gel shift activity (Fig. 2B, lanes 1 and 3). E2F-1 DNA binding activity decayed as cells progressed further into the S and G₂ phases (Fig. 2B, lanes 5 and 7, respectively), consistent with earlier results that linked the disappearance of E2F-1 DNA binding function in late S/G₂ to the appearance of cyclin A kinase at that time (21–23). Like the effect seen before in exponentially growing cells, UV treatment led to a significant (~3-fold) increase in E2F-1 DNA binding activity at all stages throughout S phase (Fig. 2B and C). Similar results were

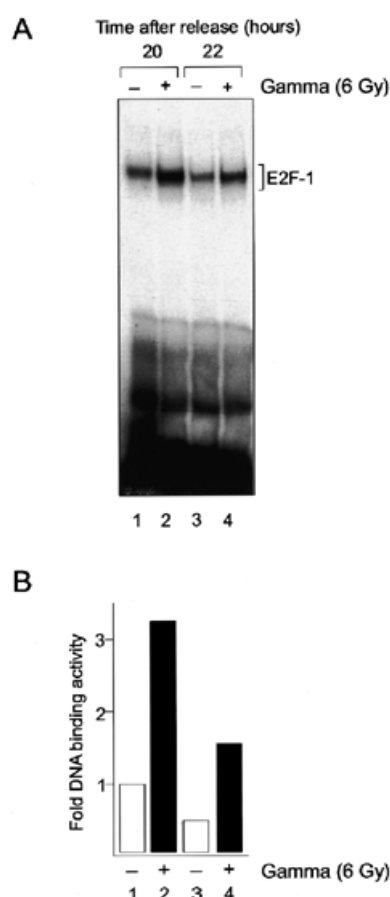


Figure 3. Activation of E2F-1 DNA binding function by γ -irradiation. (A) T98G cells were synchronized and processed exactly as described in the legend to Figure 2B, except that at indicated time points cells were exposed to 6 Gy of γ -irradiation instead of UV. (B) E2F-1 DNA binding activity shown in (A) was quantified as described in Figure 2C.

obtained when cell synchrony was achieved by mimosine or hydroxyurea block release (data not shown).

To determine whether other types of DNA damaging agents, in particular γ -irradiation, produce a similar increase in E2F-1 DNA binding activity, early S phase synchronized T98G cells were either left untreated or were treated with γ -irradiation at a dose of 6 Gy and subjected to the E2F-1 IP gel shift assay. Similar to the results obtained with UV, γ -irradiation led to an ~3-fold increase in immunoprecipitable E2F-1 DNA binding activity compared with untreated control cells (Fig. 3A and B, compare lanes 2 and 4 with 1 and 3). These results collectively suggest that the responses provoked by these two distinct DNA damaging agents involve an increase in E2F-1 DNA binding function.

The significance of these observations arises from the fact that to date, all biological activities of E2F-1 depend on its ability to function as a sequence-specific DNA binding element. Thus, with the observation that the amount of E2F-1 DNA binding activity increases significantly in response to distinct types of DNA damage, it seems fair to conclude that increased amounts of E2F-1 DNA binding activity affect the expression of one or more genes at the level of transcription. Indeed, existing evidence suggests that the expression of at least one known target gene of

E2F, ribonucleotide reductase (RNR), is up-regulated in response to DNA damage (24). RNR catalyzes the direct reduction of ribonucleotides to the corresponding deoxyribonucleotides, thereby providing the necessary precursors for DNA replication and efficient DNA repair. Given the results shown in this report, one might speculate that E2F-1 contributes, at least in part, to the activation of RNR transcription in response to DNA damage, thereby facilitating efficient DNA repair. At present, we cannot determine whether the increase in E2F-1 DNA binding activity in response to DNA damage reported here also translates into enhanced transactivation function of E2F-1. However, this is difficult to test because E2F reporter assays allow one to measure the collective activity of E2F only. Finally, a role for E2F-1 in the enaction of DNA damage checkpoint responses may be related to its apoptosis-inducing activities. At least in certain experimental settings, it was shown that the induction of apoptosis by E2F-1 does not require its transactivation function but is dependent on the ability of E2F-1 to bind DNA (14,15). Given these results and those shown here, one can imagine that the increase in E2F-1 DNA binding activity seen in response to DNA damage could directly translate into an enhanced tendency of damaged cells to undergo apoptosis.

E2F-1 function is controlled at multiple levels, including at the level of transcription, turnover, phosphorylation and interactions with other proteins (4). In an effort to understand the mechanism underlying the increase in E2F-1 DNA binding activity in response to DNA damage, we examined whether there might be changes in the levels of E2F-1 protein as a consequence of DNA damage checkpoint activation. T98G cells were synchronized and treated with UV- or γ -irradiation as before and whole cell extracts were prepared and analyzed by immunoblotting using antibodies specific for E2F-1. Exposure of T98G cells to either UV- or γ -irradiation was found to markedly increase the levels of E2F-1 protein compared with untreated cells (Fig. 4A and B, upper panel, respectively). No such effect was seen for either cyclin A (Fig. 4A, lower) or E2F-4 (Fig. 4B, lower panel) when analyzed in parallel. These data suggest that the increase in E2F-1 protein levels in response to DNA damaging agents employed here is specific. In addition, these results suggest a relationship between the DNA damage-associated increase in E2F-1 protein levels and an increase in E2F-1-dependent DNA binding activity, at least in the cell types under investigation. However, we cannot exclude other mechanisms, e.g. phosphorylation/dephosphorylation reactions, which might contribute, in part, to the rise in E2F-1 DNA binding activity after DNA damage. In addition, whether the increase in E2F-1 protein levels observed here is a result of enhanced transcription of the E2F-1 gene itself or is a reflection of an increase in E2F-1 protein stability, or both, remains to be determined. However, there are certainly interesting parallels between these observations and the known relationship between p53 and DNA damage. Specifically, p53 protein is stabilized in response to various DNA damaging agents and this stabilization appears to result from reduced p53 ubiquitination (25). E2F-1, too, is regulated by ubiquitin-mediated proteolysis (26–28). However, the relevant components mediating E2F-1 turnover have not yet been elucidated. It will be possible to investigate this issue only when the mechanism underlying E2F-1 degradation is fully understood.

The observations reported here imply E2F-1 as a participant in the DNA damage response pathway. Further exploration of this pathway should help to elucidate how the DNA damage-associated

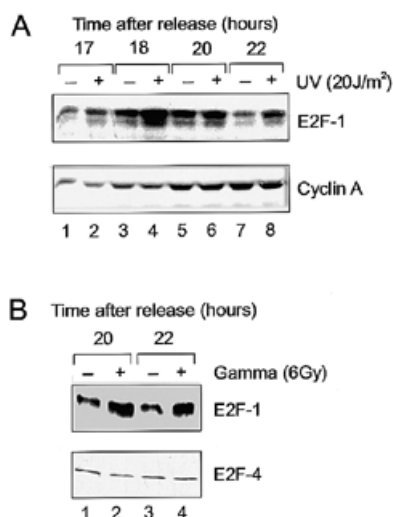


Figure 4. DNA damage-induced increase in E2F-1 protein levels. (**A** and **B**) Aliquots of whole cell extracts of T98G cells analyzed in Figures 2B and 3A for E2F-1 DNA binding activity were subjected to immunoblot analysis using either anti-E2F-1 mAb PC (upper panel), anti-cyclin A antibodies (**A**, lower panel) or E2F-4 antibodies (**B**, lower panel). Note the relative increase in E2F-1 protein levels compared with control in response to DNA damage.

increase in E2F-1 DNA binding activity is linked to the behavior of at least some of the known regulators of the DNA damage checkpoint.

ACKNOWLEDGEMENTS

We thank the members of our laboratory for many helpful discussions. We are thankful to P. Müller for synthesis of oligonucleotides and F. Fischer for synthesis of peptides. Special thanks go to the Kantonsspital Basel for the use of the γ -irradiation source. We also thank F. Reymond and J. Lisztwan for careful and critical reading of the manuscript. W.K. is a START fellow and is supported by the Swiss National Science Foundation (SNF). B.H. is supported by a predoctoral grant from the SNF.

REFERENCES

- Elledge, S.J. (1996) *Science*, **274**, 1664–1671.
- Hartwell, L.H. and Kastan, M.B. (1994) *Science*, **266**, 1821–1828.
- Levine, A. (1997) *Cell*, **88**, 323–331.
- Dyson, N. (1998) *Genes Dev.*, **12**, 2245–2262.
- Xu, G., Livingston, D.M. and Krek, W. (1995) *Proc. Natl Acad. Sci. USA*, **92**, 1357–1361.
- Singh, P., Wong, S. and Hong, W. (1994) *EMBO J.*, **13**, 3329–3338.
- Johnson, D.G., Ohtani, K. and Nevins, J.R. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 12823–12827.
- Johnson, D.G., Schwarz, J.K., Cress, W.D. and Nevins, J.R. (1993) *Nature*, **365**, 349–352.
- Kowalik, T.F., DeGregori, J., Schwarz, J.K. and Nevins, J.R. (1995) *J. Virol.*, **69**, 2491–2500.
- Qin, X.Q., Livingston, D.M., Kaelin, W.G. and Adams, P. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 10918–10922.
- Shan, B. and Lee, W.-H. (1994) *Mol. Cell. Biol.*, **14**, 8166–8173.
- Wu, X. and Levine, A.J. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 3602–3606.
- Nip, J., Strom, D.K., Fee, B.E., Zambetti, G., Cleveland, J. and Hiebert, S.W. (1997) *Mol. Cell Biol.*, **17**, 1049–1056.
- Hsieh, J.-K., Fredersdorf, S., Kouzarides, T., Martin, K. and Lu, X. (1997) *Genes Dev.*, **11**, 1840–1852.
- Phillips, A.C., Bates, S., Ryan, K.M., Helin, K. and Vousden, K.H. (1997) *Genes Dev.*, **11**, 1853–1863.
- Yamasaki, L., Jacks, T., Bronson, R., Goillot, E., Harlow, E. and Dyson, N.J. (1996) *Cell*, **85**, 537–548.
- Field, J.S., Tsai, F.-Y., Kuo, F., Zubiaga, A.M., Kaelin, W.G., Livingston, D.M., Orkin, S.H. and Greenberg, M.E. (1996) *Cell*, **85**, 549–561.
- Lisztwan, J., Marti, A., Sutterluety, H., Gstaiger, M., Wirbelauer, C. and Krek, W. (1998) *EMBO J.*, **17**, 368–383.
- Krek, W., Livingston, D.M. and Shirodkar, S. (1993) *Science*, **262**, 1557–1560.
- Leone, G., DeGregori, J., Yan, Z., Jakoi, L., Ishida, S., Williams, R.S. and Nevins, J.R. (1998) *Genes Dev.*, **12**, 2120–2130.
- Krek, W., Ewen, M.E., Shirodkar, S., Arany, Z., Kaelin, W.G., Jr and Livingston, D.M. (1994) *Cell*, **78**, 161–172.
- Dynlacht, B.D., Flores, O., Lees, J.A. and Harlow, E. (1994) *Genes Dev.*, **8**, 1772–1786.
- Xu, M., Sheppard, K.A., Peng, C.Y., Yee, A.S. and Piwnicka-Worms, H. (1994) *Mol. Cell. Biol.*, **14**, 8420–8431.
- Filatov, D., Bjorklund, S., Johansson, E. and Thelander, L. (1996) *J. Biol. Chem.*, **271**, 23698–23704.
- Maki, C.G., Huibregtse, J.M. and Howley, P.M. (1996) *Cancer Res.*, **56**, 2649–2654.
- Hateboer, G., Kerkhoven, R.M., Shvarts, A., Bernards, R. and Beijersbergen, R.L. (1996) *Genes Dev.*, **10**, 2960–2970.
- Hofmann, F., Martelli, F., Livingston, D.M. and Wang, Z. (1996) *Genes Dev.*, **10**, 2949–2959.
- Campanero, M.R. and Flemington, E.K. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 2221–2226.